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Novel pro-oxidant activity assay for polyphenols, vitamins C and E using a modified CUPRAC method

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ABSTRACT

In this study, a direct assay, a modified CUPRAC (Cupric Ion Reducing Antioxidant Capacity) method, is developed to determine transition metal ion (Cu(II))-catalyzed pro-oxidant activity of polyphenolic compounds, vitamins C and E, and herbal samples in the presence of proteins containing thiol groups. Since transition metal ion-catalyzed pro-oxidant activity of phenolics is usually initiated with the reduction of the metal to lower oxidation states (as a prerequisite of Fenton-type reactions), this method involves the reduction of copper(II) ions to copper(I) by polyphenolic compounds (simultaneously giving rise to reactive species), binding of the formed Cu(I) to egg white protein –SH groups, and liberation of copper(I)-neocuproine (Cu(I)-Nc) chelate (showing maximum absorbance at 450 nm) by treating the incubation product with a neocuproine–ammonium acetate mixture. The proposed method is validated against atomic absorption spectrometric (AAS) determination of protein-bound copper and protein carbonyl assay of oxidative stress. The proposed assay is faster and more specific than the carbonyl assay, and uses low-cost reagents and equipment. Pro-oxidant activity (*i.e.* proportional to absorbance) varies linearly over a relatively wide range with concentration, as opposed to the reciprocal correlations (*i.e.* linear regression of 1/(pro-oxidant activity) versus 1/concentration) of other similar assays. The pro-oxidant activity order of the tested antioxidant compounds in terms of 'Quercetin Equivalent Pro-oxidant Activity' (QREPA) coefficients is: gallic acid > epicatechin > quercetin≈catechin > α-tocopherol > rosmarinic acid > trolox > caffeic acid > ascorbic acid.

1. Introduction

Aerobic organisms generate reactive oxygen species (ROS) such as hydroxyl radicals ($^{\bullet}OH$), superoxide anion radicals ($O_2^{\bullet-}$), peroxyl radicals ($ROO^{\bullet}$) etc. during normal metabolism. Under oxidative stress conditions resulting from an imbalance between pro-oxidants and antioxidants, excessively produced ROS can cause harmful effects on living cells, resulting in irreparable damage to cellular macromolecules such as lipids, proteins, nucleic acids [1] eventually leading to chronic diseases such as cardiovascular and neurodegenerative diseases, hypertension, diabetes mellitus and cancer [2].

Dietary phytophenolics comprising more than 4,000 flavonoids [3] have been recognized as beneficial antioxidants that can scavenge ROS (*i.e.* $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$, and $^1O_2$), and certain structural requirements such as *o*-dihydroxy (catechol) structure in the B ring, 2,3-double bond in conjugation with a 4-keto function, and additional presence of both 3- and 5-hydroxyl groups for maximal radical scavenging and metal ion chelation are generally accepted to be responsible for high antioxidant activity of flavonoids [4]. However, phytophenolics also have the potential to act as pro-oxidants under specific conditions [5]. For example, high doses of α-tocopherol are poorly-effective at decreasing levels of lipid peroxidation in humans [6]. As regards ascorbic acid, it is well known that it acts as a pro-oxidant rather than an antioxidant in the presence of transition metal ions. This is because ascorbic acid reduces transition metal ions ($Me^{(n+1)+}+e^- \rightarrow Me^{n+}$) and generates hydrogen peroxide through autoxidation, which drives production of hydroxyl radicals *via* the Fenton reaction [7]. Furthermore it is well established by *in vitro* experiments that ascorbic acid is reactive with free iron and produces the ascorbate radical, while causing oxidative damage to biomolecules. The reduction potentials of Fe^{3+} (0.77 V) and ascorbic acid (0.25 V) easily allow the formation of ascorbate radical and Fe^{2+}. Therefore, *in vitro* ascorbic acid can exert pro-oxidant effects by converting Fe^{3+} into Fe^{2+}, which in turn reacts with H_2O_2 to generate $^{\bullet}OH$. Iron–ascorbate mixtures have been shown to stimulate free radical damage to DNA, lipids and proteins *in vitro* [8]. In addition, flavonoids and dihydroxycinnamic acids can nick DNA *via* production of radicals in the presence of O_2 and Cu(II), the latter being one of the most redox active metal ions present in cells. Both the antioxidant and the copper-initiated pro-oxidant activities of a flavonoid depend

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upon the number of hydroxyl substitutions in its backbone structure, *i.e.* the more hydroxyl substitutions, the stronger the antioxidant and pro-oxidant activities [9]. The conjugation between rings A and B is also important for the copper-initiated pro-oxidant action of a flavonoid [9]. Broadly speaking, the antioxidant/pro-oxidant activity of phytophenolics can depend on such factors as metal-reducing potential, chelating behavior, pH, and solubility characteristics [10].

In the presence of Cu(II), pro-oxidant activity of polyphenolic compounds was predicted to proceed *via* formation of $^{\bullet}OH$ by the following series of reactions:

$$PhOH+Cu^{2+} \rightarrow PhO^{\bullet}+Cu^{+}+H^{+} \quad (1)$$

$$PhO^{\bullet}+O_2 \rightarrow PhO^{+}+O_2^{\bullet-} \quad (2)$$

$$PhOH+O_2^{\bullet-}+H^{+} \rightarrow PhO^{\bullet}+H_2O_2 \quad (3)$$

$$2O_2^{\bullet-}+2H^{+} \rightarrow H_2O_2+O_2 \quad (4)$$

$$Protein\text{–}SH+Cu^{+} \rightarrow Protein\text{–}S\text{–}Cu^{I}+H^{+}\ (partly) \quad (5)$$

$$Protein\text{–}S\text{–}Cu^{I}+H_2O_2 \rightarrow Protein\text{–}S\text{–}Cu^{II}+{}^{\bullet}OH+OH^{-} \quad (6)$$

$$Protein\text{–}S\text{–}Cu^{II}+{}^{\bullet}OH \rightarrow Damaged\ protein+Cu^{2+} \quad (7)$$

The initial oxidation of phenolics (*e.g.*, catechols) by Cu^{2+} generates a semiquinone radical: $PhO^{\bullet}$ (reaction (1)) that can react with O_2 to form $O_2^{\bullet-}$ (reaction (2)). This reaction has an autocatalytic character since $O_2^{\bullet-}$ will oxidize the parent compound to regenerate the semiquinone and H_2O_2 (reaction (3)). H_2O_2 can also be formed by the disproportionation of $O_2^{\bullet-}$ (reaction (4)) [10,11]. Cu^{+} ions (essentially formed from reaction (1)) partly bind to proteins containing thiol groups (reaction (5)), and copper-bound protein may further react with H_2O_2 to generate new $^{\bullet}OH$ *via* the Fenton-type reaction (reaction (6)) which eventually leads to protein damage (reaction (7)). These series of reactions (1)–(7) were exploited in this work for the development of a modified CUPRAC method measuring pro-oxidant activity.

Proteins undergoing oxidative attack with transition metal ion catalysis give rise to carbonyl derivatives *via* a variety of mechanisms that include fragmentation and amine oxidation (*e.g.*, of side-chain amine groups on several amino acids), and consequently, protein carbonyls are commonly used as a marker of protein oxidation in cells and tissues. Protein carbonyls are generally measured spectrophotometrically using 2,4-dinitrophenylhydrazine (DNPH), having an absorbance maximum at 370 nm and a molar absorption coefficient of $2.20 \times 10^4\ M^{-1}\ cm^{-1}$ [12–14]. Various polyphenolic compounds, including catechins, were shown to cause protein carbonyl formation in proteins *via* their pro-oxidant actions [15]. The formation of protein carbonyls *via* oxidative deamination under aerobic conditions (without transition metal ions) was higher for catechins having a galloyl group than for the corresponding catechins lacking a galloyl group in the B-ring, and in addition, carbonyl formation in HSA was higher for pyrogallol-bearing catechins than for the corresponding catechol-bearing catechins in the B-ring [15]. Possible interferences to the DNPH assay can be summarized as: (i) non-enzymatic glycation of proteins may add carbonyl groups onto amino acid residues; (ii) plant and protein extracts showing significant absorbance at 370 nm (*e.g.*, hemoglobin, myoglobin) give rise to problems in quantification of carbonyl content; (iii) nucleic acids (*e.g.*, in plant extracts [16]) also contain carbonyl groups that react with DNPH, causing positive errors [17].

Although there is a wide variety of methods in literature to measure antioxidant activity, there are very few assays to measure pro-oxidant activity, and the existing ones are indirect, *i.e.* are usually based on observing the loss of protection of an oxidizable substrate undergoing oxidative attack in the presence of the test compound. One of the rare examples of a direct assay for the measurement of oxidative capacity of human plasma has recently been given by Mehdi and Rizvi who have modified the original DMPD antioxidant activity measurement by using plasma as an oxidant (free radical generator) itself and producing a pink color with DMPD (due to the oxidized radical: $DMPD^{\bullet+}$), constituting a good example of how antioxidants (existing in human plasma along with oxidants and iron as transition metal ion catalyst) may behave as pro-oxidants under certain conditions [18]. Therefore a more direct assay, the CUPRAC (Cupric Ion Reducing Antioxidant Capacity) assay originally introduced for antioxidant capacity measurement [19], was modified for the first time in this work to determine transition metal ion (Cu(II))-catalyzed pro-oxidant activity of polyphenolic compounds, vitamins C and E, and herbal samples in the presence of proteins containing thiol groups. This assay operationally defines pro-oxidant activity as the transition metal ion reducing ability of antioxidant compounds to their lower oxidation states to produce reactive species *via* Fenton-type reactions. In this assay, Cu(I) species produced from antioxidant reduction of cupric ions simultaneously give rise to reactive species and bind to egg white proteins through reactions (1)–(7), and the protein-bound cuprous ions are either colorimetrically measured after liberation with neocuproine (as the colored cuprous-neocuproine chelate) or determined by AAS in the protein solution dissolved with a suitable buffer. The protein carbonyl method originally developed as a marker of oxidative stress was used for validating the developed assay.

2. Experimental

2.1. Reagents and instrumentation

The following chemical substances of analytical reagent grade were supplied from the corresponding sources: neocuproine (2,9-dimethyl-1,10-phenanthroline), (−)epigallocatechin gallate, (−) epicatechin, quercetin, gallic acid, absolute ethanol, hydrochloric acid, methanol, sodium dodecyl sulfate (SDS) and urea: Sigma (Steinheim, Germany); naringin, *p*-coumaric acid, caffeic acid, rosmarinic acid, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), vitamin E, and ascorbic acid (AA): Aldrich (Steinheim, Germany); copper(II) chloride dihydrate, ammonium acetate, $NaH_2PO_4 \cdot 2H_2O$, and tris (tris(hydroxymethyl)aminomethane): Merck (Darmstadt, Germany); (+)catechin: Fluka (Buchs, Switzerland); Na_2HPO_4, $CuSO_4$, and trichloroacetic acid (TCA): Riedel-de Haën (Steinheim, Germany). The herbal plants were supplied from Malatya Pazari A.S. of Istanbul.

The absorbances were measured with a Perkin Elmer Lambda 35 UV–vis spectrophotometer using a pair of matched quartz cuvettes of 1 cm thickness and a Varian SpectrAA 220 atomic absorption spectrometer using a copper hollow cathode lamb. The pH measurements were made with the aid of a HI 221 Calibration Check Microprocessor pH-meter using a glass electrode. A Velp Scientifica vortex apparatus was used to stir the incubation solutions. An Electromag M4812P centrifuge apparatus was used for separation of the protein residues.

2.2. Preparation of solutions

$CuCl_2$ solution, 1.0×10^{-2} M, was prepared by dissolving 0.4262 g $CuCl_2 \cdot 2H_2O$ in distilled water, and diluted to 250 mL. Ammonium acetate buffer at pH = 7.0, 1.0 M, was prepared by dissolving 19.27 g NH_4Ac in distilled water and diluted to 250 mL. Neocuproine (Nc) solution, 7.5×10^{-3} M, was prepared daily by dissolving 0.078 g Nc in absolute ethanol, and diluted to 50 mL with ethanol. Copper(II) sulfate solution, 1.0×10^{-3} M, was

prepared by dissolving 0.0125 g $CuSO_4$ in distilled water, and diluted to 50 mL. The $Na_2HPO_4/NaH_2PO_4 \cdot 2H_2O$ buffer solution, 0.2 M, at pH = 7.4, was prepared in distilled water. All polyphenolic compounds and vitamin E solutions were freshly prepared in absolute ethanol at 1.0×10^{-3} M concentration. Ascorbic acid solution, 1.0×10^{-3} M, was prepared daily by dissolving 0.0125 g AA in distilled water, and diluted to 50 mL. Trichloroacetic acid (TCA) at 10% (by mass) was prepared in distilled water. 2,4-Dinitrophenylhydrazine solution, 1.0×10^{-2} M, was prepared by dissolving 0.1981 g DNPH in 0.2 M HCl, and diluted to 100 mL. Tris solution, 5.0×10^{-2} M, was prepared by dissolving 1.5142 g tris in distilled water. SDS solution, 2%, was prepared by dissolving 5 g SDS in distilled water. Urea solution, 8 M, was prepared by dissolving 120.12 g urea in distilled water. The protein dissolution buffer that was adjusted to pH = 6.8 with 2 M HCl and diluted to 250 mL with distilled water, contained 5.0×10^{-2} M tris, 2% SDS, and 8 M urea. This buffer was used for dissolving TCA-precipitated egg white proteins in the experiments. The egg white was completely separated from the yolk, weighed (37.6126 g), and suspended in distilled water to make a 250 mL final solution.

2.3. Herbal plant infusion

Two-gram amount of the herbal plant (sage, green tea, mint, elderberry, linden and rosemary) was steeped in 50 mL MeOH/H_2O (4:1, v/v) for 10 min in an ultrasonic bath. The extract was obtained after filtering through a filter paper, then through a microfilter.

2.4. General procedures

2.4.1. Recommended procedure (modified CUPRAC assay)

To a test tube were added 1.0 mL of phosphate buffer (pH 7.4), 1.0 mL of 1.0 mM copper(II) solution, 1.0 mL of 1.0 mM polyphenolic compound solution (or 1.0 mL aliquot of herbal plant extract in 80% MeOH/water (v/v)), 1.0 mL of egg white solution and 1.0 mL of distilled water (or 1.0 mL of 1.0 mM rosmarinic or caffeic acid for standard addition). The final mixture at 5.0 mL total volume was vortexed for 5 min and let to stand at room temperature for exactly 25 min. At the end of this time, 1.0 mL of 10% TCA solution was added to the incubation solution and protein was precipitated. Then the mixture was centrifuged for 10 min at 4000 rpm, the upper liquid phase decanted, and the precipitate washed three times with distilled water. To the precipitate were added 1.0 mL of Nc solution and 1.0 mL of NH_4Ac buffer, stirred, and diluted to 4.1 mL with distilled water. The mixture was then filtered through a filter paper and the absorbance at 450 nm (A_{450}) was recorded against a CUPRAC reagent blank. Pro-oxidant activity was calculated using the difference between absorbance values recorded in the presence and absence of polyphenolic compounds.

The scheme for pro-oxidant activity measurement of polyphenols is summarized as:

$$1\ \text{mL pH 7.4 Phosphate Buffer} + 1\ \text{mL } Cu^{2+} + 1\ \text{mL AOx} + 1\ \text{mL Protein} + 1\ \text{mL } H_2O$$

$$\xrightarrow[25\ \text{min incubation}]{5\ \text{min vortex}} 1\ \text{mL TCA} \xrightarrow{10\ \text{min centrifuge}} 1\ \text{mL Nc} + 1\ \text{mL } NH_4Ac + 2.1\ \text{mL } H_2O$$

$$V_{total} = 4.1\ \text{mL}$$

The standard calibration curve of each polyphenolic compound was constructed in this manner as absorbance versus molar concentration, and the molar absorptivity of the modified CUPRAC assay for each antioxidant was found from the slope of the calibration line concerned.

2.4.2. Atomic absorption spectrometry (AAS)

AAS method is based on adding protein dissolution buffer (instead of CUPRAC reagent) to incubation product and determining the copper passed into solution by AAS.

2.4.3. Protein carbonyl detection assay

Carbonyl detection assay is based on the measurement of the absorbance at 370 nm wavelength of dinitrophenylhydrazones (DNP) formed from the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl groups emerging from protein oxidation [13,20]. To a test tube were added 1 mL of phosphate buffer (pH 7.4), 1.0 mL of 1.0 mM copper(II) solution, 1.0 mL of 1.0 mM polyphenolic compound solution (or 1.0 mL aliquot of herbal plant extracts in 80% MeOH/water (v/v)), 1.0 mL of egg white solution and 2.0 mL of distilled water. The final mixture at 6.0 mL total volume was stirred and incubated for 30 min in a water bath kept at 37 °C. At the end of this period, 1.0 mL of 10.0 mM DNPH solution was added and incubated for 30 min at room temperature. Then, 1.0 mL of 10% TCA solution was added to incubation solution and protein was precipitated. The mixture was centrifuged for 10 min at 4000 rpm, the upper liquid phase was decanted, and the precipitate was washed three times with distilled water. To the precipitate, 1.0 mL protein dissolution buffer was added and diluted to 4.1 mL with distilled water. To another test tube were added all incubation solutions, the final mixture at 6.0 mL total volume was stirred and not incubated; DNPH and TCA solutions were immediately added and subsequent procedures performed in the same way. The test tube containing the final mix was filtered through a filter paper and the absorbance at 370 nm (A_{370}) was recorded against water. Pro-oxidant activity was calculated using the difference between absorbance values recorded in the presence and absence of polyphenolic compounds; plant extracts were measured with compensation for the initial A_{370} values not resulting from protein oxidation. For carbonyl detection assay, there was no significant difference between measurements obtained by washing of the protein precipitate with either distilled water or ethanol.

2.5. Statistical analysis

Descriptive statistical analyses were performed using Excel software (Microsoft Office 2003) for calculating the means and the standard error of the mean. Results were expressed as the mean ± standard deviation (S.D.). Using SPSS software for Windows (version 13), the data were evaluated by two-way ANalysis Of VAriance (ANOVA) [21].

3. Results and discussion

Polyphenols are generally recognized as antioxidants, but also act as pro-oxidants inducing DNA degradation in the presence of transition metal ions such as copper through the generation of reactive oxygen species (ROS), and such copper-mediated pro-oxidant activity of polyphenols may cause preferential cytotoxicity towards cancer cells showing elevated copper levels [22]. In this regard, DNA-bound Cu(II) may be especially toxic *via* redox cycling with polyphenols, as it may form a ternary complex with DNA and polyphenols and be reduced to Cu(I), the reoxidation of which by molecular oxygen generates ROS causing DNA breaks [23]. Hadi et al. hypothesized that the mechanism for the chemopreventive/anticancer properties of polyphenols involves mobilization of endogenous (*e.g.*, chromatin-bound) copper of cancer cells *via* redox-cycling, inducing their apoptosis [24]. Seemingly, some antitumor agents such as apogossypolone (*i.e.* a less toxic derivative of the polyphenolic aldehyde gossypol) may cause oxidative

DNA breakage in cancer cells [25], and genistein may show preferential cytotoxicity against breast cancer cells by mobilization of endogenous copper ions [26] rich in tissues, cells and serum of various malignancies. Endogenous copper is basically responsible for the polyphenol-induced degradation of DNA, as it was demonstrated by Khan et al. that copper-overloaded lymphocytes—upon treatment with polyphenols like epigallocatechin gallate, genistein and resveratrol—showed increased DNA breakage, which was inhibitable by membrane-permeable copper-chelator neocuproine but not by membrane-impermeable copper chelator bathocuproine disulfonate [27].

In the development of the modified CUPRAC assay for pro-oxidant activity measurement of antioxidant compounds, copper and protein were selected to simulate real cases of pro-oxidant action where reactive species are generated through transition metal ion (such as endogenous copper)-initiated reactions damaging biological macromolecules. Copper(II)→copper(I) reduction was utilized because Fenton-type reactions require lower oxidation states of metals. Although iron is considerably more abundant in biological systems, the major ions in the nucleus are copper and zinc. Copper is a highly redox-active metal ion present in chromatin, and is closely associated with DNA bases, particularly guanine [28]. The Cu(I) produced from Cu(II) with antioxidants not only generates reactive species but at the same time binds to partly damaged protein –SH groups. Egg white as a low-cost protein source was found to be superior to BSA in this regard since its thiol groups are more abundant, enabling a more clear-cut separation of the produced Cu(I) without loss of reproducibility.

Total pro-oxidant activity (TPA) values of synthetic mixtures were calculated as mM QR equivalent, and of herbal plant extracts as μmol QR g^{-1} equivalent. The proposed methodologies of protein-bound Cu(I) measurement were validated by comparison of the results with those of the reference carbonyl detection assay. Carbonyl detection with DNPH was selected due to its simplicity and versatility in spite of limited specificity. The compounds used for comparison were chosen to represent different classes of antioxidants and polyphenols, such as gallic acid (simple phenolic acid), rosmarinic acid, caffeic acid, *p*-coumaric acid (hydroxycinnamic acids), (+)catechin, (−)epicatechin, (−)epigallocatechin gallate (flavanols), quercetin (flavonol), naringin (flavanone), ascorbic acid, α-tocopherol and trolox (other antioxidants). As both the developed CUPRAC assay and AAS measurement determine the cuprous ion content at copper-binding protein, their results were expected to be more comparable.

3.1. Determination of the molar absorption coefficients, linear ranges, and correlation coefficients of polyphenolic compounds

According to modified CUPRAC, AAS and carbonyl detection assays, correlation coefficients (r), molar absorption coefficients (ε), and linear ranges were calculated from calibration equations of polyphenolic compounds dissolved in EtOH (Tables 1–3). Although naringin and *p*-coumaric acid responded to modified CUPRAC, AAS and carbonyl detection assays, their linear working ranges could not be determined due to low precision of experimental data, probably arising from the relatively higher reduction potentials of their $ArO^{\bullet}$/ArOH couples making Cu(II)→Cu(I) reduction more difficult. The linear concentration range of epigallocatechin gallate could be determined only with respect to the carbonyl detection assay, but not to the modified CUPRAC and AAS assays (Tables 1–3), the possible reasons of which need further investigation. Substitution of the 3-OH group with a bulkier galloyl group could increase the torsion angle of ring B with the rest of the molecule, causing a loss of coplanarity and decreased conjugation [29]. Since overall conjugation of a flavonoid is closely related to its electron transfer ability [9], both antioxidant and transition metal-induced pro-oxidant activity of epigallocatechin gallate compared to those of other catechins may be adversely affected. Although it may appear at first glance that, based on the findings of Ishii et al. [15], protein carbonyl formation would be expected to be higher for the pyrogallol-type catechin, epigallocatechin gallate, than the corresponding catechol-type catechin, epicatechin, it should be remembered that the indicated study involved incubation of proteins with polyphenols under aerobic conditions without transition metal ions, and that the carbonyl-forming oxidative deamination reactions involved quinone binding to protein amino-groups [15]. On the other hand, the primary structure of proteins undergoes significant degradation under the copper-catalyzed oxidative conditions of the current study, which may not enable protein-quinone binding prior to protein carbonyl formation. Furthermore, the relatively lower pro-oxidant activity of epigallocatechin gallate may also be attributed to molecular size, since bulky phenolic polymers like tannins were shown to exhibit little or no pro-oxidant activity, as opposed to many small phenolics which are pro-oxidants [30].

3.2. Determination of quercetin equivalent pro-oxidant activity (QREPA) coefficients of polyphenolic compounds

QREPA (QueRcetin Equivalent Pro-oxidant Activity) coefficient is defined as the millimolar quercetin equivalent pro-oxidant activity of a 1.0 mM solution of the polyphenolic compound under investigation, therefore, QREPA is dimensionless. The molar absorptivities of the concerned polyphenolic compounds were found from the slopes of the calibration equations of the modified CUPRAC, AAS and carbonyl detection assays (Tables 1–3), and divided by the molar absorptivity of quercetin under identical conditions to obtain the QREPA coefficients (Table 4).

$$QREPA\ coefficients = \frac{\varepsilon_{AOx}}{\varepsilon_{QR}}\ (AOx: antioxidant) \quad (8)$$

Due to reproducibility problems stated before, both the calibration equations/linear concentration ranges and the corresponding ε values for *p*-coumaric acid, naringin, and (−)epigallocatechin gallate could not be precisely found (the only exception was the response of epigallocatechin gallate to the carbonyl assay). The order of $QREPA_{Modified\text{-}CUPRAC}$ coefficients of polyphenolic compounds was: gallic acid > epicatechin > catechin > α-tocopherol > rosmarinic acid > trolox > caffeic acid > ascorbic acid. The order of $QREPA_{AAS}$ coefficients was: gallic acid > epicatechin > catechin > rosmarinic acid > α-tocopherol > caffeic acid > trolox > ascorbic acid. Both modified CUPRAC and AAS methods marked catechins as the second most potent pro-oxidants in the test system, in accordance with literature reports stating that catechol ring-containing flavonoids have pro-oxidant activity and form electrophilic quinone/quinone methide intermediates which bind to DNA, protein, and GSH [31]. On the other hand, the order of $QREPA_{Carbonyl\text{-}Detection}$ coefficients was: rosmarinic acid > epicatechin > gallic acid > catechin > epigallocatechin gallate > caffeic acid = α-tocopherol > ascorbic acid > trolox (Table 4). The modified CUPRAC and AAS assays were more compatible among each other than the modified CUPRAC–carbonyl detection pair of assays (with correlation coefficients (r) of 0.9016 and 0.6096, respectively), because the latter are based on different mechanisms and different products are measured.

Naringin, *p*-coumaric acid and epigallocatechin gallate showed poor pro-oxidant activity. Naringin is a flavanone glycoside attached to the A-ring, and flavonoid glycosides are known to possess less antioxidant and pro-oxidant behavior than the corresponding aglycons. Moreover, it was previously shown that flavanones have much lower copper-initiated pro-oxidant activity than the analogic flavones with the same number of hydroxyl groups

Table 1
The calibration equations, molar absorption coefficients and linear ranges of the tested polyphenolic compounds with respect to the modified CUPRAC assay.

Polyphenolic compound	Calibration equation	Molar absorption coefficient ($L\ mol^{-1}\ cm^{-1}$)	Linear range ($mol\ L^{-1}$)
Simple phenolic acid			
Gallic acid (GA)	$A=447.1c+0.113$ $r=0.9632$	447.1	1.04×10^{-4}–2.30×10^{-3}
Hydroxycinnamic acids			
Rosmarinic acid (ROS)	$A=228.8c+0.077$ $r=0.6862$	228.8	3.64×10^{-4}–4.65×10^{-3}
Caffeic acid (CFA)	$A=173.3c+0.085$ $r=0.9181$	173.3	4.33×10^{-4}–6.09×10^{-3}
p-Coumaric acid (COU)	–	–	–
Flavanols			
(+)Catechin (CT)	$A=342.0c+0.078$ $r=0.9446$	342.0	2.39×10^{-4}–3.10×10^{-3}
(−)Epicatechin (EC)	$A=388.3c+0.091$ $r=0.9343$	388.3	1.78×10^{-4}–2.70×10^{-3}
(−)Epigallocatechin gallate (EGCG)	–	–	–
Flavonol			
Quercetin (QR)	$A=346.7c+0.072$ $r=0.9582$	346.7	2.55×10^{-4}–3.08×10^{-3}
Flavanone			
Naringin (NG)	–	–	–
Others			
Ascorbic acid (AA)	$A=120.2c+0.046$ $r=0.9884$	120.2	9.52×10^{-4}–9.11×10^{-3}
α-Tocopherol (α-Toc)	$A=276.8c+0.043$ $r=0.9830$	276.8	4.21×10^{-4}–3.96×10^{-3}
Trolox (TR)	$A=204.8c+0.049$ $r=0.9789$	204.8	5.41×10^{-4}–5.33×10^{-3}

Table 2
The calibration equations, molar absorption coefficients and linear ranges of the tested polyphenolic compounds with respect to atomic absorption spectrometry (AAS).

Polyphenolic compound	Calibration equation	Molar absorption coefficient ($L\ mol^{-1}\ cm^{-1}$)	Linear range ($mol\ L^{-1}$)
Simple phenolic acid			
Gallic acid	$A=666.4c+0.140$ $r=0.8919$	666.4	3.00×10^{-5}–1.50×10^{-3}
Hydroxycinnamic acids			
Rosmarinic acid	$A=392.5c+0.149$ $r=0.7781$	392.5	2.67×10^{-5}–2.52×10^{-3}
Caffeic acid	$A=286.6c+0.098$ $r=0.9341$	286.6	2.17×10^{-4}–3.64×10^{-3}
p-Coumaric acid	–	–	–
Flavanols			
(+)Catechin	$A=424.1c+0.112$ $r=0.9373$	424.1	1.12×10^{-4}–2.42×10^{-3}
(−)Epicatechin	$A=432.4c+0.158$ $r=0.8982$	432.4	5.09×10^{-6}–2.27×10^{-3}
(−)Epigallocatechin gallate	–	–	–
Flavonol			
Quercetin	$A=530.1c+0.094$ $r=0.9691$	530.1	1.24×10^{-4}–1.97×10^{-3}
Flavanone			
Naringin	–	–	–
Others			
Ascorbic acid	$A=200.3c+0.011$ $r=0.9920$	200.3	7.44×10^{-4}–5.64×10^{-3}
α-Tocopherol	$A=314.1c+0.098$ $r=0.9514$	314.1	1.99×10^{-4}–3.32×10^{-3}
Trolox	$A=227.3c+0.070$ $r=0.9534$	227.3	3.95×10^{-4}–4.71×10^{-3}

Table 3
The calibration equations, molar absorption coefficients and linear ranges of the tested polyphenolic compounds with respect to the carbonyl detection assay.

Polyphenolic compound	Calibration equation	Molar absorption coefficient ($L\ mol^{-1}\ cm^{-1}$)	Linear range ($mol\ L^{-1}$)
Simple phenolic acid			
Gallic acid	$A=274.4c+0.088$ $r=0.9891$	274.4	2.64×10^{-4}–3.83×10^{-3}
Hydroxycinnamic acids			
Rosmarinic acid	$A=352.4c+0.137$ $r=0.9866$	352.4	6.41×10^{-5}–2.84×10^{-3}
Caffeic acid	$A=135.6c+0.095$ $r=0.7996$	135.6	4.79×10^{-4}–7.71×10^{-3}
p-Coumaric acid	–	–	–
Flavanols			
(+)Catechin	$A=248.0c+0.086$ $r=0.9490$	248.0	2.98×10^{-4}–4.25×10^{-3}
(−)Epicatechin	$A=303.2c+0.132$ $r=0.9356$	303.2	9.30×10^{-5}–3.32×10^{-3}
(−)Epigallocatechin gallate	$A=142.0c+0.084$ $r=0.9918$	142.0	5.38×10^{-4} -7.44×10^{-3}
Flavonol			
Quercetin	$A=463.1c+0.090$ $r=0.9921$	463.1	1.50×10^{-4}–2.27×10^{-3}
Flavanone			
Naringin	–	–	–
Others			
Ascorbic acid	$A=96.9c+0.011$ $r=0.9935$	96.9	1.54×10^{-4}–1.16×10^{-2}
α-Tocopherol	$A=135.9c+0.078$ $r=0.9970$	135.9	6.02×10^{-4}–7.81×10^{-3}
Trolox	$A=71.1c+0.087$ $r=0.8553$	71.1	1.03×10^{-3}–1.48×10^{-2}

[9]. Gallic acid has higher activity than both rosmarinic acid and caffeic acid, because it has three hydroxyl groups, and both antioxidant and pro-oxidant activities are known to increase with the number of phenolic –OH groups [9].

3.3. Total pro-oxidant activity (TPA) of synthetic mixtures

Binary combinations of polyphenolic compound solutions were prepared by adding 1.0 mL of the concerned polyphenols to an incubation mixture solution; their pro-oxidant activities were experimentally found as mM QR equivalents, *i.e.* QR equivalent TPA of the mixture was calculated by dividing the observed absorbance (A_{450}) to the molar absorptivity of quercetin, and compared with those theoretically found (Fig. 1). The theoretical quercetin equivalent pro-oxidant activity of a synthetic mixture solution was calculated by summing up the absorbances of concerned polyphenols and dividing by the molar absorptivity of QR. Data in Fig. 1 show that, with the exception of GA+EC mixture, the theoretical and experimental TPA values were generally comparable.

Table 4
The QREPA (QueRcetin Equivalent Pro-oxidant Activity) coefficients of the tested polyphenolic compounds with respect to the modified CUPRAC, AAS and carbonyl detection assays.

Polyphenolic compound	QREPA values with respect to Modified CUPRAC assay	QREPA values with respect to AAS assay	QREPA values with respect to Carbonyl Detection assay
Simple phenolic acid			
Gallic acid	1.29	1.26	0.59
Hydroxycinnamic acids			
Rosmarinic acid	0.66	0.74	0.76
Caffeic acid	0.50	0.54	0.29
p-Coumaric acid	–	–	–
Flavanols			
(+)Catechin	0.99	0.80	0.54
(−)Epicatechin	1.12	0.82	0.65
(−) Epigallocatechin gallate	–	–	0.31
Flavonol			
Quercetin	1.00	1.00	1.00
Flavanone			
Naringin	–	–	–
Others			
Ascorbic acid	0.35	0.38	0.21
α-Tocopherol	0.80	0.59	0.29
Trolox	0.59	0.43	0.15

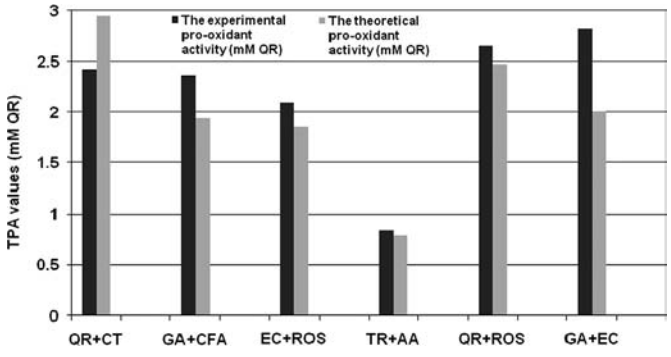

Fig. 1. The theoretical and experimental total pro-oxidant activities of synthetic mixtures with respect to the modified CUPRAC assay (concentrations of synthetic mixtures; quercetin–catechin 10^{-3} M, gallic acid–caffeic acid, epicatechin–rosmarinic acid, trolox–ascorbic acid, gallic acid–epicatechin 10^{-4} M and quercetin–rosmarinic acid 5.0×10^{-4} M).

3.4. Total pro-oxidant activity (TPA) of herbal plant extracts

Sage, green tea, mint, elderberry, linden and rosemary were studied as real samples. The modified CUPRAC, AAS and carbonyl detection assays were applied to extracts in 80% methanol–water (v/v). The pro-oxidant activities of herbal plant extracts with respect to these assays were expressed as μmol QR g^{-1}. When the original carbonyl assay was applied without compensation for initial values, it did not basically correlate with the modified CUPRAC assay. However, when the TPA value of the non-incubated procedure was subtracted from that of the incubated one, the modified CUPRAC and carbonyl detection assays were well correlated (Fig. 2). This may be attributed to the non-specific response of the carbonyl assay to various constituents of plant extracts that react with DNPH, causing positive errors [17].

The two-way ANalysis Of VAriance (ANOVA) comparison by the aid of *F*-test of the mean-squares of 'between-treatments' (*i.e.* modified CUPRAC and carbonyl detection test results in Fig. 2) and of residuals [21] for a number of real samples enabled to conclude that there was no significant difference between treatments. In other words, the TPA values found with the two procedures for a given herbal plant extract were alike at 95% confidence level ($F_{exp}=0.710$, $F_{crit}=4.545$, $F_{exp}<F_{crit}$ at $P=0.05$). Thus, the proposed methodology was validated for real samples.

The modified CUPRAC assay was applied to herbal plant extracts with standard addition of rosmarinic acid and caffeic acid. The theoretical and experimental total pro-oxidant activities (TPA) of extracts were expressed as μmol QR g^{-1}.

$$\text{The Theoretical TPA} = \frac{(A_{Extr}+A_{AOx})}{\varepsilon_{QR}} \times \frac{\text{Total volume}}{\text{Taken volume}} \times \frac{\text{Extract volume}}{\text{g Herbal plant}} \quad (9)$$

$$\text{The Experimental TPA} = \frac{\text{Absorbance}}{\varepsilon_{QR}} \times \frac{\text{Total volume}}{\text{Taken volume}} \times \frac{\text{Extract volume}}{\text{gHerbal plant}} \quad (10)$$

The theoretical and experimental total pro-oxidant activities found using the modified CUPRAC method were basically additive and coherent (Table 5). Comparing the theoretical and experimental total pro-oxidant activities for rosmarinic acid and caffeic acid standard additions, the correlation coefficients (*r*) were 0.9649 and 0.7985, respectively.

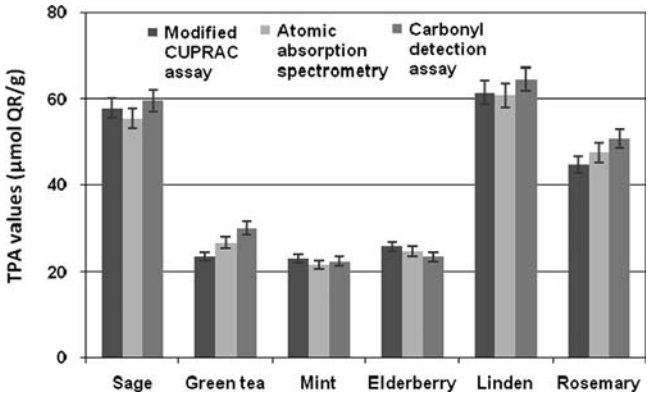

Fig. 2. The total pro-oxidant activities of herbal plant extracts with respect to the modified CUPRAC, AAS and carbonyl detection assays (the ANOVA comparison between modified CUPRAC and carbonyl detection assays: $P=0.05$, $F_{exp}=0.710$, $F_{crit\ (table)}=4.545$, $F_{exp}<F_{crit\ (table)}$. Data presented as (mean ± SD), $N=3$).

Table 5
The theoretical and experimental total pro-oxidant activities of herbal plants to Modified CUPRAC assay (mean ± SD, $N=3$ data points).

Standard addition to herbal plant	The theoretical total pro-oxidant activity (μmol QR g^{-1})	The experimental total pro-oxidant activity (μmol QR g^{-1})
Sage extract+ROS	114.2 ± 1.0	121.3 ± 3.0
Sage extract+CFA	123.3 ± 0.7	102.5 ± 2.0
Green tea+ROS	62.9 ± 2.6	70.2 ± 2.4
Green tea+CFA	81.9 ± 0.7	71.2 ± 0.5
Linden+ROS	117.1 ± 1.7	123.4 ± 2.6
Linden+CFA	135.9 ± 0.9	112.5 ± 2.2
Mint+ROS	71.6 ± 2.6	93.8 ± 2.0
Mint+CFA	90.6 ± 0.7	101.8 ± 2.3

4. Conclusion

Although plant polyphenols and vitamins have beneficial health effects due to their antioxidant properties, they may act as pro-oxidants and show toxic effects depending on concentration, pH, and transition metal ion used for inducing pro-oxidant behavior. Pro-oxidant activity has been operationally defined as the transition metal ion reducing ability of antioxidant compounds to their lower oxidation states which may produce reactive species *via* Fenton-type reactions. In accordance with this definition, a modified CUPRAC assay was developed to determine Cu(II)-catalyzed pro-oxidant activity of polyphenolic compounds, vitamins C and E, and herbal extracts in the presence of proteins containing thiol groups. The utilized protein-copper complex is in conformity to the model involved in the biological pro-oxidant action of polyphenols generating reactive species with endogenous copper through redox-cycling. This method is believed to fill an important literature gap, as there are no direct, reliable and widely accepted pro-oxidant activity measurement methods in the literature. Pro-oxidant activity (*i.e.* proportional to absorbance) varied linearly over a relatively wide range with concentration, as opposed to the reciprocal correlations (*i.e.* linear regression of 1/(pro-oxidant activity) versus 1/concentration) of other similar assays [9]. Although the proposed method was validated against the widely used carbonyl assay using DNPH colorimetry, the latter is not specific [17] and is adversely affected by many interferences. The proposed assay only takes 30 min, and therefore is faster than the carbonyl assay. The modified CUPRAC assay uses less reagents which are commonly available at low cost, and it can diversely be applied to antioxidant compounds. It works in the visible (450 nm) range of the electromagnetic spectrum, and therefore is less susceptible to interferences. It is suitable for rather less-equipped conventional laboratories not using sophisticated instruments like ESR.

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